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Research article

Immune stimulatory effect of *Nigella sativa* in healthy animal models: A systematic review and meta-analysis

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ABSTRACT

The immune-modulatory effects of black seeds (*Nigella sativa* seeds, NSS) are well documented, but the overall *in vivo* impact of this important natural medicinal product on immune system function has yet to be established. Here we systematically reviewed and meta-analyzed the effects of NSS on humoral [serum titers of immunoglobulins including IgG, IgM, anti-Newcastle virus disease (anti-NDV), and sheep red blood cell antigen (anti-SRBC)] and cellular immunity [total white blood cell (WBC) count and percentages of monocytes, lymphocytes, basophils, neutrophils, and eosinophils] in healthy animals. The PubMed, ScienceDirect, Web of Science, and Scopus databases were searched according to predefined eligibility criteria. Meta-analyses were performed to estimate the final effect size using RevMan software. Seventeen animal studies were eligible for analysis. For humoral immunity, the overall pooled effect size (ES) of NSS on serum titers of IgM and anti-NVD antibodies was not significantly different [mean difference (MD) 75.27, 95% CI: −44.76 to 195.30, $p = 0.22$ ($I^2 = 89\%$, $p = 0.003$), and −0.01, 95% CI: −0.27 to 0.25, $p = 0.94$ ($I^2 = 74\%$, $p = 0.02$), respectively]. However, NSS significantly increased serum titers of IgG and anti-SRBC antibodies [MD 3.30, 95% CI: 2.27 to 4.32, $p = 0.00001$ ($I^2 = 0\%$, $p = 0.97$), and 1.15, 95% CI: 0.74 to 1.56, $p = 0.00001$ ($I^2 = 0\%$, $p = 0.43$), respectively]. For cellular immunity, the ES of NSS on WBCs, monocytes, and lymphocytes were not significantly different

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[MD 0.29, 95% CI: −0.55 to 1.13, $p = 0.50$, ($I^2 = 14\%$, $p = 0.32$), - 0.01, 95% CI: −0.45 to 0.44, p = 0.97 ($I^2 = 0\%$, $p = 0.77$), and 4.73, 95% CI: −7.13 to 16.59, $p = 0.43$, ($I^2 = 99\%$, $p = 0.00001$), respectively]. In conclusion, black seeds enhance humoral immunity in healthy animals but do not affect cellular immunity.

1. Introduction

Black seeds or black cumin seeds, or habbatussauda in Arabic (botanical name: *Nigella sativa* L.*;* family: *Ranunculaceae*), is a spice native to Southwest Asia [1–5] rich in proteins, fats, carbohydrates, vitamins (A, B1, B2, B3, and C), minerals (calcium, potassium, selenium, copper, phosphorus, zinc, and iron), crude fiber, and cellulose [1,3,4]. *Nigella sativa* seeds (NSS) also contain essential oils, volatile oils, and fatty acids (e.g., linoleic, oleic, dihomolinoleic, eicodadienoic, myristic, palmitoleic, linoleic, linolenic, and arachidonic acids) along with several phytosterols including cholesterol, campesterol, β-sitosterol, Δ5-avenasterol, Δ7-stigmasterol, and Δ7-avenasterol [1,3,4]. NSS also contain isoquinoline alkaloids (e.g., nigellicimine and nigellicimine N-oxide), pyrazole alkaloids (e.g., nigellidine and nigellicine), and terpenes (e.g., thymoquinone, carvacrol, 4-terpineol, t-anethol, sesquiterpene longifolene, α-pinene, and thymol) [1,3,4]. However, the versatile pharmacological characteristics of NSS are mainly due to their quinine components, particularly thymoquinone [6].

Given their complex composition, NSS are thought to exert significant bioactivity and are widely consumed across the world as a food supplement [7] and to treat illnesses [1,2,5] in several countries, including Arab nations, Asia, Africa, and Europe [6]. There have been many studies of the therapeutic effects of NSS in several diseases [1,5,8–12], particularly for immune disorders [13]. Studies performed during the recent novel coronavirus disease 2019 (COVID-19) pandemic further suggested that NSS may positively affect

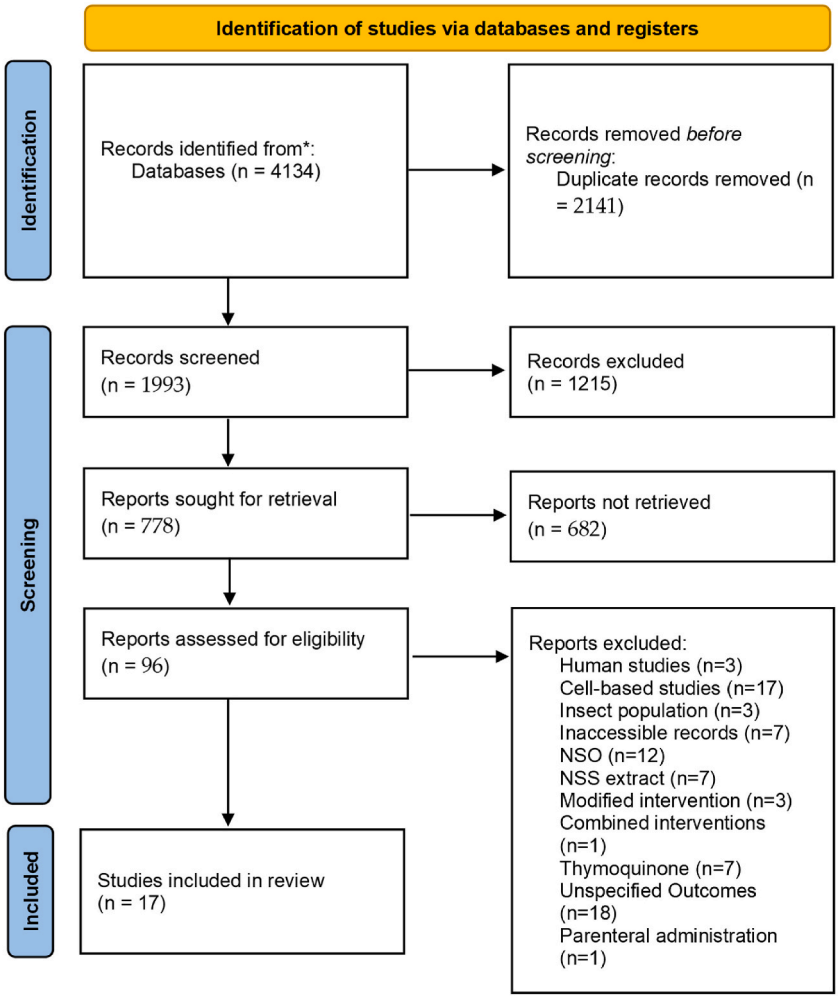


Fig. 1. Flow chart according to updated PRISMA checklist 2020. Abbreviations: NSS: *Nigella sativa* seeds, NSO: *Nigella sativa* oil.

immune health [4,13].

White blood cells (WBCs) and their subtypes (monocytes, lymphocytes, neutrophils, basophils, and eosinophils) form the cellular component of immunity and inflammatory reactions in response to injury and pathogens. Total WBCs counts and their subtypes are often used as outcome measures to estimate cellular immunity [14]. Lymphocytes are activated by dendritic cells to differentiate specialized T and B cells [15,16] that mediate adaptive cellular immune responses [15,16]. Monocytes participate in monocyte-related innate immune responses [17]. In terms of humoral (antibody-mediated) immunity, serum immunoglobulin M (IgM) increases during the early primary immune response and serum IgG increases during secondary immune responses [18], so serum IgG and IgM titers are often measured to estimate humoral immunity outcomes.

There have been several narrative reviews on the effects of NSS on immune responses [3,4,10]. Additionally, there have been systematic reviews of the preclinical and clinical efficacy of NSS in diabetes mellitus, respiratory disorders (e.g., asthma and bronchitis), rheumatism, headache, back pain, paralysis, inflammation, hypertension, oxidative stress, nephrotoxicity, inflammation, and hepatorenal function [5,6,19–23]. However, there have not been any systematic reviews and meta-analyses of the effects of NSS on immunity in healthy animal models, even though robust preclinical evidence could provide important insights into the mechanism of action of NSS for clinical translation. This knowledge gap prompted us to critically appraise studies examining the efficacy of NSS on immunity in healthy experimental animals, given that a robust summary of the preclinical evidence of the immunomodulatory effects of NSS in experimental systems would be extremely useful for the planning and design of human studies. We asked whether NSS modulates humoral and cellular immunity in healthy animals compared with untreated healthy animal controls and conducted a systematic review and meta-analysis to answer this question.

2. Results

2.1. Identification and screening of studies

The studies identified in different databases and study selection are detailed in Supplementary Table S1 and Fig. 1.

2.2. Selected studies

Seventeen studies were eligible and relevant to the intervention (i.e., NSS) and the prespecified outcomes in healthy animals [24–40]. All 17 studies were assessed for internal validity (risk of bias (RoB) assessment). All studies were considered in the qualitative literature synthesis depending on the efficacy of the highest dose (Fig. 1).

2.3. Assessment of risk of bias

2.3.1. Across all studies

Approximately 20% of included studies neglected to randomize the animals upon allocation to the intervention and control groups. Baseline characteristics and outcomes reporting were optimally implemented (100%), whereas no study conducted blinding during the assignment of animals into groups, administration of treatments to animals, and performing outcome measurements (100%). Attrition bias could not be evaluated adequately in 90% of included studies, because the statistical methods did not indicate how missing values or dead animals were addressed. Similarly, other sources of bias could not be evaluated in 53% of included studies due to a lack of evidence on funding details or conflicts of interest (Fig. 2).

2.3.2. Within each study

Seventeen included studies showed a low risk of bias and were eligible as evidence in the systematic literature review (Fig. 3).

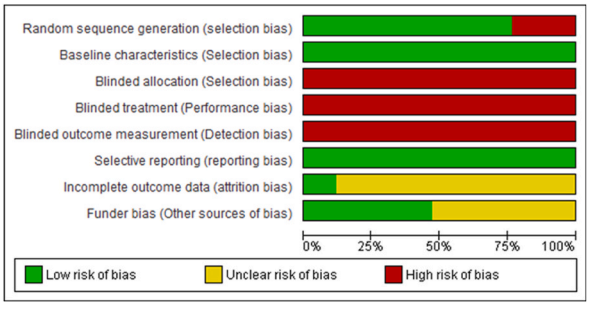


Fig. 2. Risk of bias assessment across studies. The figure was generated using RevMan after applying the CYRCLE RoB tool for assessing risk of bias in animal studies (Supplementary Table S2).

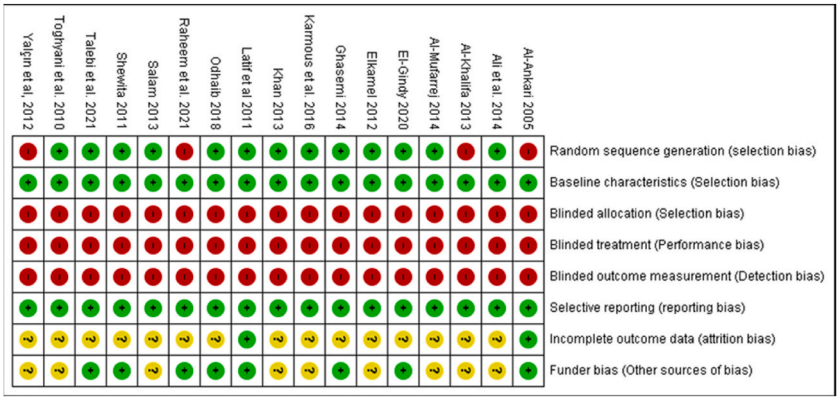


Fig. 3. Risk of bias assessment within studies. Red indicates a high risk of bias, green is a low risk, and yellow indicates an unclear risk. The figure was generated using RevMan after applying the CYRCLE RoB tool for assessing risk of bias in animal studies (Supplementary Table S2).

2.3.3. Study characteristics and consistency

2.3.3.1. Population. The animal models were consistent; only healthy animals were recruited into studies. However, there was heterogeneity, because the animals were not pooled from a similar population (studies included chicken, rabbits, fish, and lambs) (Supplementary Table S3).

2.3.3.2. Intervention. The interventions were consistent, with only (powdered or crushed) NSS considered. Additionally, the interventions were consistent because the feeding techniques used in the studies were identical (NSS mixed in with the usual diet, allowing animals to feed *ad libitum*). However, there was heterogeneity in dose and treatment follow-up duration (Supplementary Table S3).

2.3.3.3. Comparators. All controls were placebos (*ad libitum* basal diet). The baselisne characteristics of the animals in the control groups (species, gender, age, health status, and genetic background) were balanced with the corresponding interventional groups. Like the interventional groups, controls were subjected to identical exposure conditions (pre-treatment measures, housing conditions, feeding technique, and duration of treatment) (Supplementary Table S3).

2.3.3.4. Outcomes. The included studies used the same outcomes to measure cellular or humoral immunity (Supplementary Table S3).

2.3.3.5. Study design. The studies were conducted between 2011 and 2021 in different countries including Pakistan, Saudi Arabia, Egypt, Iran, Malaysia, and Indonesia. All studies followed a parallel interventional model (Supplementary Table S3).

2.3.3.6. Literature synthesis

2.3.3.6.1. Qualitative synthesis.

- Humoral immunity

Two studies reported that NSS significantly increased serum IgG titers [28,32], one study reported a significant increase in serum IgM titers [32], and another study reported a non-significant change in serum IgM titers [28].

Four studies reported that NSS significantly increased serum titers of anti-SRBC antibodies [26,32,37,40], while one study reported that NSS failed to induce a significant change in serum anti-SRBC titers [26].

Four studies reported that NSS significantly increased serum titers of anti-NDV antibodies [31,33,34,39]. Conversely, four studies reported that NSS failed to induce significant changes in serum anti-NDV titers [25,29,30,38]. In contrast, one study reported that NSS could significantly decrease serum anti-NDV antibody titers [24].

- Cellular immunity

Four studies reported that NSS significantly increased the total WBC count [26,27,32,36], while one study reported a significant decrease in the total WBC count [38]. Conversely, seven studies reported that NSS failed to significantly change the total WBC count [25,26,28–30,35,37].

Concerning monocytes, one report indicated that NSS significantly increased the monocyte percentage [38], while three reports demonstrated that NSS failed to induce a significant change in monocyte percentage [32,35,37].

One study reported that NSS significantly increased lymphocyte percentage [27], while another study reported a significant decrease in lymphocytes percentage [38] and two studies reported that NSS failed to induce a significant change in lymphocytes percentage [32,37].

Only one study reported that NSS failed to induce a significant change in neutrophil percentage [32]. One report indicated that NSS significantly increased basophil percentage [38], while another study reported that NSS did not cause a significant change [37].

One study reported that NSS significantly increased eosinophil percentage [38], but three studies reported that NSS did not induce a significant change in eosinophil percentage [32,35,37].

2.3.3.6.2. Quantitative meta-analysis. • Publication bias

Visualization of funnel plots for either humoral (Fig. 4a) or cellular (Fig. 4b) immunity showed no risk of publication bias.

- Efficacy of NSS on humoral immunity

For serum IgM titers, the two eligible studies [28,32] showed that the overall pooled effect size of NSS was not significantly different [mean difference (MD) = 75.27, 95% CI: −44.76 to 195.30, $p = 0.22$, with substantial heterogeneity ($I^2 = 89\%$, $p = 0.003$)] (Fig. 5a). However, these two reports [28,32] showed that the overall pooled effect size of NSS on serum IgG titers was significantly different in favor of the NSS group [MD = 3.30, 95% CI: 2.27 to 4.32, $p = 0.00001$, with zero heterogeneity ($I^2 = 0\%$, $p = 0.97$] (Fig. 5b).

For serum anti-SRBC titers, six reports were included [26,31,32,37,40], among them two reports by Al-Khalifa, Al-Nasser [26]. The overall pooled effect size of NSS on serum anti-SRBC titers was significantly different in favor of the NSS group [MD = 1.15, 95% CI: 0.74 to 1.56, $p = 0.00001$, with zero heterogeneity ($I^2 = 0\%$, $p = 0.43$] (Fig. 6a).

For serum anti-NDV titers, three reports were included [25,30,33]. The overall pooled effect size of NSS on serum anti-NDV titers was not significantly different [MD = - 0.01, 95% CI: −0.27 to 0.25, $p = 0.94$, with substantial heterogeneity ($I^2 = 74\%$, $p = 0.02$] (Fig. 6b).

- Efficacy of NSS on cellular immunity

For the total WBC count, 11 reports were included [25–30,32,35,36,38], among them two reports by Al-Khalifa, Al-Nasser [26]. The meta-analysis showed that the overall pooled effect size of NSS on total WBC count was not significantly different [MD = 1.96, 95% CI: 0.84 to 4.76, $p = 0.17$, with substantial heterogeneity ($I^2 = 100\%$, $p = 0.00001$] (Fig. 7a). Similarly, after repeating the meta-analysis, five reports were included [28–30,35,36]. The overall pooled effect size of NSS on the total WBC count was still not significantly different [MD = 0.29, 95% CI: −0.55 to 1.13, $p = 0.50$, with low heterogeneity ($I^2 = 14\%$, $p = 0.32$] (Fig. 7b).

For monocyte percentage, only two studies were included [32,35]. The results showed that the overall pooled effect size of NSS was not significantly different [MD = - 0.01, 95% CI: −0.45 to 0.44, $p = 0.97$, with zero heterogeneity ($I^2 = 0\%$, $p = 0.77$] (Fig. 8a). For

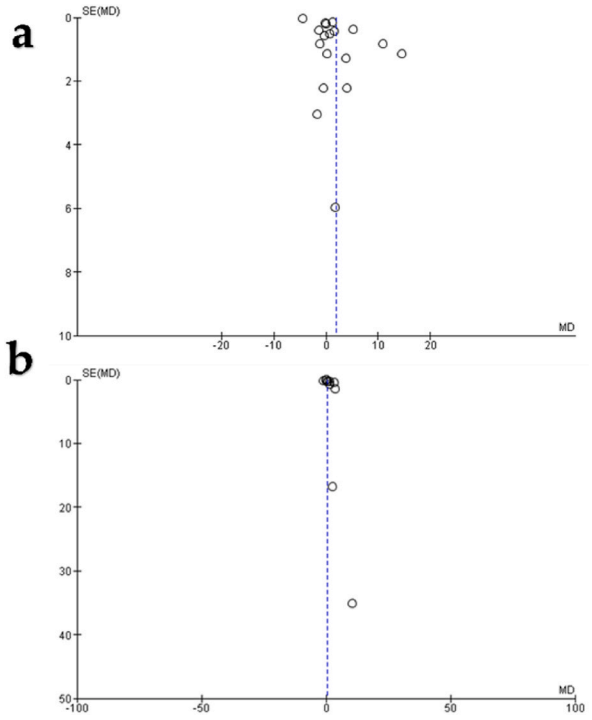


Fig. 4. Funnel plots of publication bias in humoral (a) and cellular (b) immunity at the level of meta-analyzed outcomes. MD: mean difference, SE (MD): standard error of the mean difference.

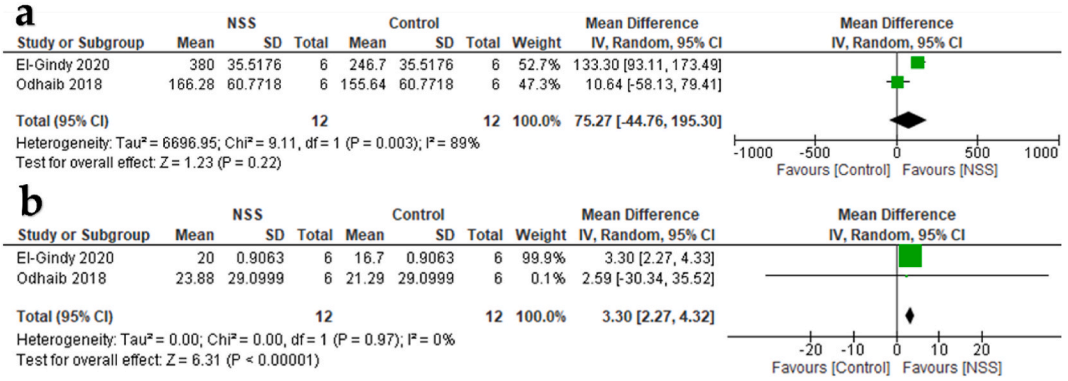


Fig. 5. (a): Forest plot of serum IgM titers; (b): forest plot of serum IgG titers. The diamond shape denotes the overall pooled effect size, SD: standard deviation, CI: confidence interval, I^2: heterogeneity percentage. NSS: *Nigella sativa* seeds. All data were meta-analyzed using a random effects model, assuming variability in animals and NSS dose across reports.

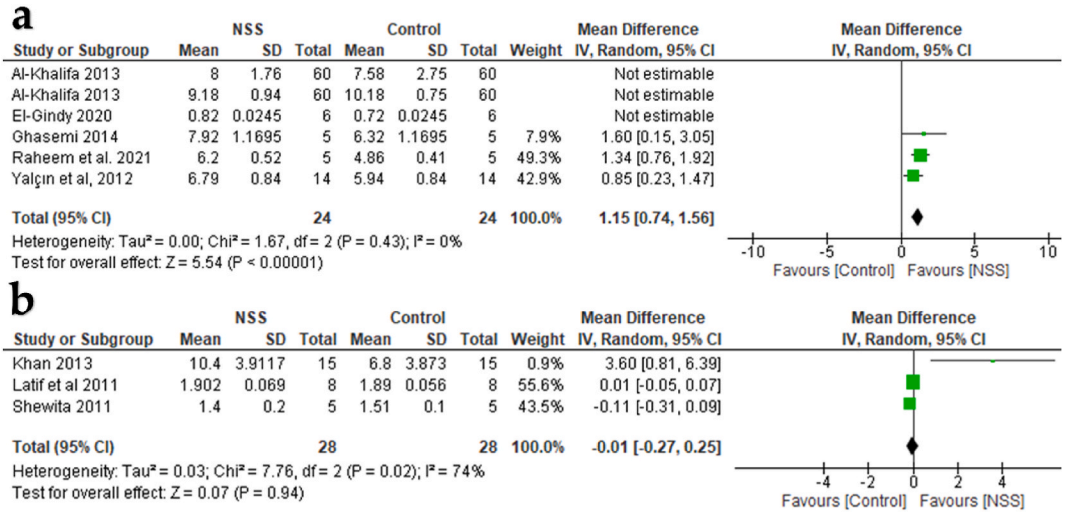


Fig. 6. (a): Forest plot of serum anti-SRBC (sheep red blood cell antigen) titers, (b): forest plot of serum anti-NDV (Newcastle disease virus) titers. NSS: *Nigella sativa* seed. The diamond shape denotes the overall pooled size effect, SD: standard deviation, CI: confidence interval, and I^2: heterogeneity percentage. All data were meta-analyzed using a random effects model, assuming variability in animals and NSS dose across reports.

lymphocyte percentage, two reports were included [27,32], which showed that the overall pooled effect size of NSS was not significantly different [MD = 4.73, 95% CI: −7.13 to 16.59, $p = 0.43$, with substantial heterogeneity ($I^2 = 99\%$, $p = 0.00001$] (Fig. 8b). For eosinophils, two reports were included [32,35], which showed that the overall pooled effect size of NSS was not significantly different [MD = −0.16, 95% CI: −0.55 to 0.22, $p = 0.41$, with substantial heterogeneity ($I^2 = 0\%$, $p = 0.88$)] (Fig. 8c). The meta-analysis was not applicable to neutrophil and basophil percentages, because only one study was available for each outcome measure.

3. Discussion

This systematic review was conducted to provide high-quality evidence on the effects of NSS on cellular and humoral immunity in healthy animals. A summary of the results of this systematic analysis is presented in Fig. 9. Seventeen studies met the eligibility criteria, which were subjected to internal validity (RoB) assessment that indicated 20% violation of randomization and 100% violation of blinding during animal selection (allocation of animals into groups), performing the study (treating animals with NSS), and detection (outcome measurement). However, these violations are highly common in preclinical studies, because randomization is not yet standard practice in animal studies [41].Additionally, assessor blinding is difficult when performing animal studies, because the same assessors are usually involved in animal selection, performing the study, and outcome measurement [41]. Moreover, most of the included studies neglected to address animal attrition (death events), and several did not report the estimator of the outcome measures and failed to report the sample size in the results. The latter drawbacks could explain why the results of some outcome measures were not included in the meta-analysis. Consequently, these systemic errors might be expected to overestimate or underestimate the effect

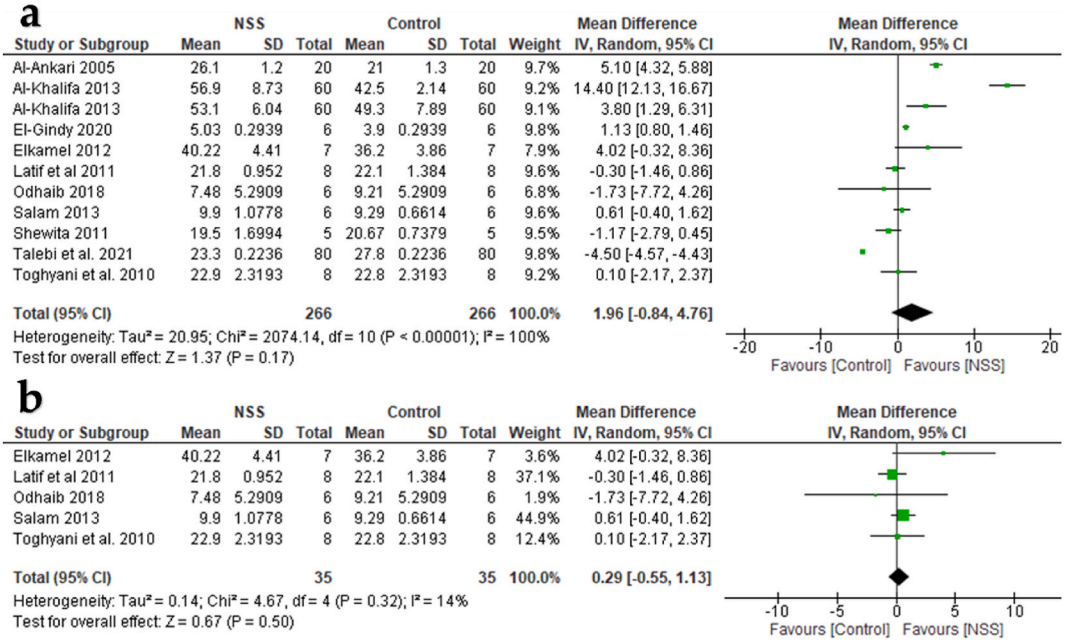


Fig. 7. Forest plot of the total WBC count. (a): Forest plot of total WBC count before applying sensitivity testing, (b): Forest plot of total WBC count after applying sensitivity testing. NSS: *Nigella sativa* seed. The diamond shape denotes the overall pooled size effect, SD: standard deviation, CI: confidence interval, and I^2: heterogeneity percentage. All data were meta-analyzed using a random effects model, assuming variability in animals and NSS dose across reports.

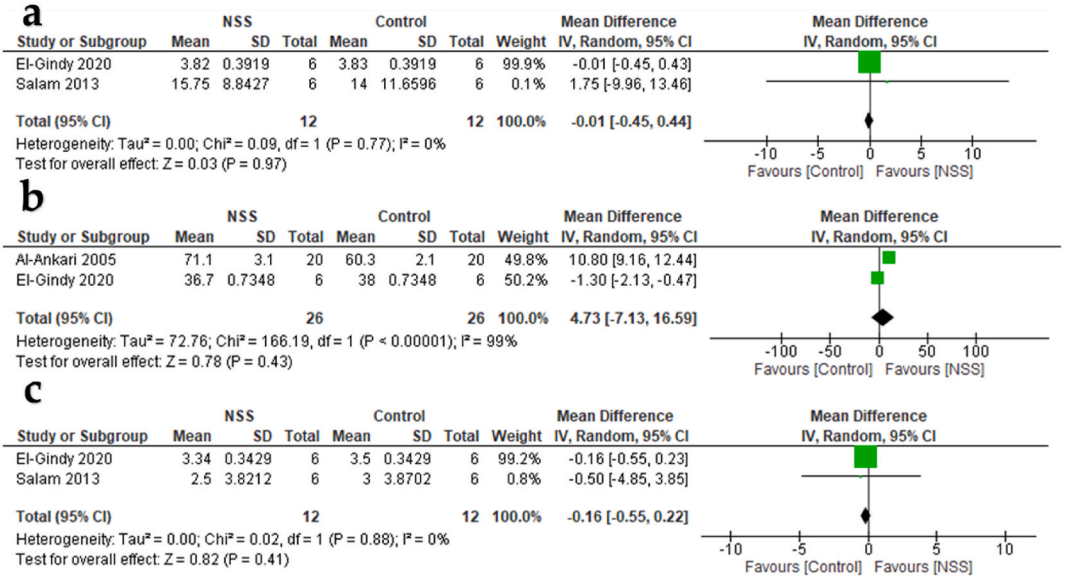
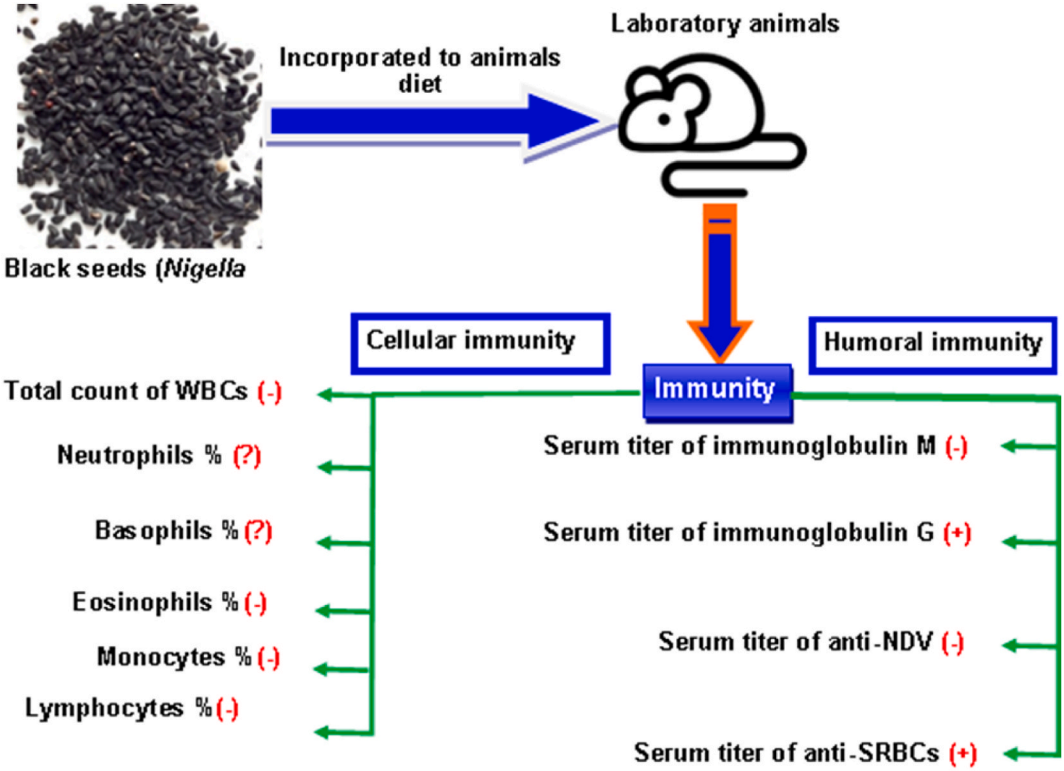


Fig. 8. Forest plots of monocyte, lymphocyte, and eosinophil percentages. (a): Forest plot of monocyte percentage, (b): Forest plot of lymphocyte percentage, (c): Forest plot of eosinophil percentage. NSS: *Nigella sativa* seed. The diamond shape denotes the overall pooled size effect, SD: standard deviation, CI: confidence interval, and I^2: heterogeneity percentage. All data were meta-analyzed using a random effects model, assuming variability in animals and NSS dose across reports.

size of the outcomes [41].

Nevertheless, we addressed these limitations by enrolling studies in the meta-analysis [42], which was performed when continuous quantitative data were available. Furthermore, the data were consistent because the included reports only investigated the effects of *ad libitum* NSS incorporated into the diets of healthy animals. The effects of animal model, treatment duration, and NSS dose heterogeneity on the evidence were minimized in the random effects model meta-analysis.



WBCs	White blood cells
%	Percentage
Anti-NDV	Antibodies against Newcastle Disease Virus
Anti-SRBCs	Antibodies against Sheep Red Blood cells antigen
?	Inadequate experimental evidence
(-)	No effect
(+)	Positive effect

Fig. 9. Schematic summary of the results of the systematic review and meta-analysis.

Only a limited number of studies reported the efficacy of NSS on serum IgG and IgM titers. Nevertheless, the pooled evidence from the meta-analysis indicated a significant increase in serum IgG titers but no significant change in serum IgG titers. Since serum IgM increases during the early primary immune response and serum IgG increases during secondary immune responses [18], this meta-analysis evidence suggests that NSS could enhance secondary humoral immunity against bacterial or viral infections in healthy animals [43].

Four studies (vs. one showing the opposite result) indicated that healthy animals exposed to SRBC antigens increased serum titers of anti-SRBC antibodies. Similarly, a meta-analysis confirmed that NSS increased serum anti-SRBC titers in healthy animals, with zero heterogeneity. Conversely, four studies showed conflicting evidence of an increase [31,33,34,39] or no change [25,29,30,38] in serum anti-NDV titers, and one study even reported a significant decrease in serum anti-NDV titers [24]. Accordingly, the evidence on the efficacy of NSS on serum anti-NDV titers is conflicting, but we note that the meta-analysis may have been imprecise due to considerable heterogeneity (74%). Based on our analysis, NSS may have an immunostimulatory effect at the level of humoral immunity by enhancing the secondary immune responses of healthy animals against viral and bacterial infections. However, further, well-designed studies with robust internal validity should now be undertaken to verify the immune potential of NSS on humoral immunity.

An increase in the number of monocytes indicates stimulation of monocyte-related innate immunity [17], while an increase in lymphocyte percentage indicates enhanced lymphocyte-dependent immunity (adaptive cellular immunity) [15,16]. Seven vs. five studies reported that NSS failed to induce significant changes in total WBC count. Our analysis indicated a non-significant difference in the total WBC count in healthy animals. Note that the number of studies available for the qualitative literature review and meta-analysis of monocytes, lymphocytes, and eosinophils was limited, and the evidence was conflicting. Indeed, there was only one report each for basophils and neutrophils in the literature, so meta-analysis of these variables was impossible. Nevertheless, we can

conclude that NSS probably fails to induce a significant cellular immune response.

In this systematic review and meta-analysis, all included studies were interventional parallel-controlled studies of healthy animals, which indicates that evidence about the immune potential of NSS and the results of the meta-analysis could be generalizable to healthy animals. The results of this systematic review will be valuable for directing future research or even clinical trials to evaluate the efficacy of NSS on cellular and humoral immunity, particularly given their long history of safe consumption by people [3,4]. On the other hand, one of the critical limitations of most of the included studies is the consideration of the total count of WBCs as a main parameter of evaluating the effect of NSS on cellular immunity. However, not each one of the white blood cells has the same importance in the cellular immunity as lymphocytes and monocytes, which constitute the backbone of the cellular immunity. Thus, future research should focus on evaluating the percentages of monocytes and lymphocytes as key parameters in evaluating the effect of NSS on the cellular immunity.

4. Conclusions

Our analysis suggests that NSS might enhance humoral but not cellular immunity in healthy animals. Further studies must be designed with robust internal validity to minimize the risks of bias and heterogeneity.

5. Materials and methods

This systematic review used the updated 2020 PRISMA checklist (Supplementary Table S5). The prospective protocol for this systematic review and meta-analysis was registered in the PROSPERO database (ID: CRD42021268472).

5.1. Search strategy

"Nigella sativa" AND "immune"; "black seed" AND "immune"; "black cumin" AND "immune"; "thymoquinone" AND "immune"; and "Nigella sativa oil" AND "immune" were used as keywords and search terms to retrieve relevant studies published between 2000 and 2022 without restriction of population, study design, type of published documents, country, or language. A pilot search strategy was implemented to robustly assess the efficiency and comprehensiveness of the keywords, and the PRESS (Peer Review of Electronic Search Strategies) checklist was followed to ensure a robust search strategy. The published literature was retrieved from the PubMed, ScienceDirect, Web of Science, and Scopus databases, and the OpenGrey, Trip Medical Database, MedNar, and ProQuest databases were searched for unpublished literature. The reference lists of published studies and publisher libraries were also searched. Two investigators implemented the search independently, while a third investigator confirmed the results and resolved discrepancies.

Table 1
Predefined eligibility criteria for relevant studies.

PICOS domains	Eligible	Ineligible
Study design	Parallel interventional model Animal-based studies • Conducted between 2000 and 2022 in any country	Human studies Cell-based studies
Population	• Animals of any type, species, sex (male or female), or strain	• Humans, cells, or insects
Health condition of interest	• Humoral and cellular immunity in healthy animals	• Unhealthy animals (diseased)
Intervention	• Black seeds only (intact, crushed, or powdered), incorporated into a diet at any treatment duration, dose level, timing, or dose frequency	• Parenteral administration • Combined interventions (e.g., standard or herbal medicinal supplements) • Chemically modified content of black seeds • Pure phytoconstituents of black seeds (e.g., thymoquinone) Black seed oil Black seed extract
Comparator	• Untreated healthy animals with identical health status and exposure conditions to those in the interventional group	• Immunostimulant therapy (drugs or herbal supplements) • Animals with unidentical health status and exposure conditions to those in the interventional group
Outcomes	• Humoral immunity through measuring serum immunoglobulins (IgM and IgG) titers, serum titers of antibodies targeting Newcastle disease virus (anti-NDV), and serum titers of antibodies targeting sheep red blood cells (anti-SRBCs) • Cellular immunity through measuring the total count of white blood cells (WBCs) as well as percentages of monocytes, lymphocytes, neutrophils, basophils, and eosinophils in blood samples	• Count of red blood cells Serum antioxidants
Additional criteria	Accessible full-text manuscripts	• Reviews, conference abstracts, books • Inaccessible full texts Retracted articles • High risk-biased studies

5.2. Study selection

After removing duplicates using EndNote reference management software, the remaining records were screened by title and abstract to retrieve related articles. The full text of relevant articles was then screened. Relevant full texts were included based on predefined eligibility criteria according to the PICOS framework (population, intervention, comparator, outcome, and study design) in response to the research question (Table 1). Two authors assessed the screened studies for eligibility, and a third opinion was sought in case of any discrepancy.

5.3. Risk of bias assessment

After selecting eligible studies, internal validity was evaluated by assessing the risk of bias at the outcome level (across all studies and within each study) by applying questions of the SYRCLE RoB tool and using RevMan v5.4 (Cochrane Collaboration, University of Oxford, UK) [44]. Six domains were evaluated for each relevant study during animal selection, performing the experiment, detecting the outcomes, attrition of animals or data for any reason, reporting the outcomes, and other sources of bias (Supplementary Table S1). An answer "low" indicated a low risk of bias, while "high" indicated a high risk of bias [45]. If the domains could not be evaluated due to inadequate information, the response was "unclear". The risk of bias was evaluated to assess its impact on the quality of evidence [45].

5.4. Data collection and study characteristics

5.4.1. Data collection strategy

Full texts were collected and encoded by assigning a code and masking the identity of the author's names and affiliations. Two independent investigators extracted and collected the data items from the texts, tables, and figures for completion in an Excel spreadsheet. A third investigator reviewed discrepancies and the accuracy of the collected data. In case of missing data, supplementary data were reviewed, or the authors were contacted by email. If the full text was inaccessible, the study was excluded.

5.4.2. Collected data

According to the PICOS criteria, in response to the research question, we collected data on:

- Study design: first author with year, country, and interventional model.
- Population data: animal types (birds, rodents, fish, non-human primates), species, age, sex (males and females), strain, health status, and the total number of animals enrolled in experimental and control groups.
- Intervention data: dose level, route of administration, dose frequency, timing, duration of treatment (time of follow-up), the vehicle of the intervention, and the number of animals per interventional group.
- Comparator data: vehicle, placebo, dose level, route of administration, dose frequency, timing, duration of treatment, and the number of animals per control group.
- Outcome data.

Serum levels of immunoglobulin M (IgM) and G (IgG) were used as outcome measures to evaluate the efficacy of NSS on humoral immunity in healthy animals compared with untreated controls measured in mg/dl. The second set of outcome measures were serum antibody titers against SRBC (sheep red blood cell antigen) and NDV (Newcastle disease virus), defined as the agglutination expressed as the $\log_2$ of the reciprocal of the highest serum dilution giving complete agglutination measured at the target time of treatment.

Several outcome measures were used to evaluate the efficacy of NSS on cellular immunity, including total white blood cell (WBC) count and percentages of monocytes, lymphocytes, neutrophils, basophils, or eosinophils. The total WBC count is reported as $x10^3$ cells/μl, while individual constituent cell types are reported as percentages.

All outcomes were continuous quantitative variables extracted from the tables or texts of the relevant records as means ± SD and sample size (n). If the units of the outcome measures were not consistent, then they were standardized using the international system conversion method. If the outcome measures were estimated as mean ± SD or confidence interval (CI), then data were standardized as mean ± SD using the RevMan v5.4 calculator.

5.4.3. Data synthesis and meta-analysis

5.4.3.1. Narrative systematic review. The evidence for each outcome from all included studies was combined within a narrative report to provide an impression of the trend of the evidence as either increasing, decreasing, or no change. The decision for this trend was made according to the magnitude of the efficacy of the highest dose level shown in most of the included studies. For each outcome, if a minimum of three or more studies reported the same outcome using a similar outcome measure, the data were pooled, and meta-analyses were performed to estimate the final effect size using RevMan.

5.4.3.2. Meta-analysis. The meta-analysis was performed by enrolling the estimators (mean ± SD and sample size) of each continuous quantitative measure in the two arms (intervention and control) for each outcome measure (humoral or cellular immunity). The mean

difference (MD) was calculated for continuous outcome measures: serum titers of IgM or IgG, anti-SRBC antibodies, or anti-NDV antibodies; total WBC count; and the percentage of monocytes, lymphocytes, neutrophils, basophils, or eosinophils (Supplementary Table S4).

The effect size (ES) for each outcome measure was expressed as the MD with 95% CIs of the positive or negative weighted average effect to determine the estimate's precision.

Random effects models were used to determine the ES with the assumption of the lowest heterogeneity between included studies (moderate heterogeneity = 50%). A random effects model was recommended because the included animal models assumed that all sampling was not from the same population. Heterogeneity was evaluated using the χ^2 test and was measured with the I^2 statistic using RevMan. For convenience, if a study measured an outcome at multiple doses, only the highest dose level was used in the meta-analysis.

Data for each outcome were standardized using identical outcome measures, units of measurement, and estimator (mean ± SD). Sensitivity testing was performed by repeating the meta-analysis after removing studies with a high risk of bias for each outcome separately. For each outcome, a funnel plot (for those including ten or more studies) was developed and visually observed for symmetry to assess publication bias before and after applying sensitivity tests. If publication bias was detected, the certainty of the evidence was minimized and not considered when drawing conclusions.

Data availability statement

All the data related to this systematic review and meta-analysis included in the manuscript supplementary files, and there is no extra data to be provided or deposited.

CRediT authorship contribution statement

Abdulsamad Alsalahi: Writing – original draft, Software, Data curation, Conceptualization. **Nian N.N. Maarof:** Project administration, Investigation, Data curation. **Mohammed A. Alshawsh:** Validation, Resources, Investigation, Data curation. **Musheer A. Aljaberi:** Software, Methodology, Investigation, Formal analysis, Data curation. **Mousa A. Qasem:** Validation, Software, Investigation, Formal analysis, Data curation. **Abdulaleem Mahuob:** Software, Project administration, Investigation, Formal analysis, Data curation. **Nassrin A. Badroon:** Software, Methodology, Formal analysis, Data curation. **Ebthag A.M. Mussa:** Validation, Resources, Data curation. **Rukman A. Hamat:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Atiyah M. Abdallah:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:ATIYEH ABDALLAH reports article publishing charges was provided by Qatar University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27390.

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